Short Communication

Restriction Fragment Length Polymorphism Typing of *Staphylococcus aureus* Strains Isolated from Bovine Mastitis and Dairy Products in Ahvaz, Iran, Using of Digested Coagulase Gene

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ABSTRACT

*Staphylococcus aureus* is a major pathogen in the transmission of diseases from animals to humans and vice-versa. Various infections, such as mastitis in cattle, sheep and goats, as well as gastroenteritis due to food poisoning in humans are the most frequent problems caused by *S. aureus*. The bacteria also lead to severe economic losses in dairy industry. A major virulence factor for the organism is encoded by the coagulase (*coa*) gene. This study aimed to assess the polymorphisms of the *coa* gene in *S. aureus* strains isolated from bovine mastitis and dairy product samples in Ahvaz, Iran. The results showed that out of 91 *S. aureus*, 80 (87.91%) isolates were positive for *coa* gene(s). In total, nine different polymerase chain reaction (PCR) products were obtained for coa-positive isolates. A single band was detected in *coa* PCR with a size ranges from 370 to 830 bp in most isolates (n=77, 96.25%). For three isolates (3.75%), two amplification products were obtained. An estimated size of 590 bp was the most frequent, as obtained for 48 (60.00%) isolates. Whereas, 370 and 830 bp PCR products were the least presented, for two (2.50%) and one (1.25%) isolate, respectively. Subsequently, for restriction fragment length polymorphism (RFLP), typing of *coa* gene and AluI restriction enzyme were used for the digestion of the products. AluI for most of PCR products generated a unique pattern; however, four PCR products (the sizes ranged 750, 670, 590, and 510 bp) generated three or more patterns. Based on AluI RFLP of *coa* gene, the isolates were classified into 23 groups. Two groups of isolates were dominant, making 45% of the total. According to the findings, one or two types of *coa* RFLP were dominant among samples that were infected with more *S. aureus* isolates belonging to different *coa* RFLP types.

Keywords: *Staphylococcus aureus*, Coagulase Gene, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism, Bovine Mastitis, Dairy Products, Iran

Typage des souches de *Staphylococcus aureus* isolées à partir de mastites bovines et de produits laitiers à Ahvaz (Iran), en utilisant le polymorphisme de la longueur des fragments de restriction du gène de la coagulase

Résumé: Staphylococcus aureus est un agent pathogène majeur dans la transmission de maladies d'animaux à l'homme et vice versa. Diverses infections, telles que les mammites chez les bétails, moutons et chèvres, ainsi que les gastro-entérites dues à une intoxication alimentaire chez l'homme, sont les problèmes les plus fréquemment causés par *S. aureus*. La bactérie entraîne également de graves pertes économiques dans l'industrie laitière. L’un des facteurs de virulence majeur pour ces microorganismes est codé par le gène de la coagulase (*coa*). Cette étude visait à évaluer les polymorphismes du gène *coa* dans les souches de *S. aureus* isolées à partir d'échantillons de mammites bovines et de produits laitiers à Ahvaz, en Iran. Les résultats ont montré que 80
INTRODUCTION

*Staphylococcus aureus* is an important pathogen causing a variety of diseases in animals and humans. Mastitis, dermatitis, udder impetigo, tick pyaemia, botryomycosis, bumble-foot, arthritis, abscess, and suppurative (pus-forming) infections are the most frequent problems caused by *S. aureus* in animals (Markey et al., 2013; Umesha and Manukumar, 2018). Staphylococcal mastitis has major impacts on both economy and animal welfare in the production of dairy ruminants, especially cows. *S. aureus* is associated with various forms of mastitis, among which subclinical mastitis is of particular importance, as it goes unnoticed and continues to affect livestock production (Suleiman et al., 2012). *S. aureus* is recognized as the main cause of food poisoning and gastroenteritis in humans worldwide. In fact, foodborne diseases are commonly caused by staphylococcal food poisoning (SFP) (Shin et al., 2016). SFP involves the consumption of dairy products, milk, and meat, particularly in traditional foods. *S. aureus* strains have been isolated frequently from SFP cases (Tsegmed et al., 2007; Hasman et al., 2010). Food poisoning due to *S. aureus* is related to the production of heat-stable enterotoxins in foodstuffs. The bacterium can shed into milk through udders infected with clinical or subclinical mastitis and environmental contamination during the handling and processing of milk and other dairy products (Morandi et al., 2010). In addition, *S. aureus* is a major nosocomial pathogenic agent, which causes pneumonia, phlebitis, meningitis, deep-seated infections (e.g., osteomyelitis and endocarditis), and skin infections, and soft tissue infections (e.g., furuncles, carbuncles, cellulitis, and abscesses) (Shopsin and Kreiswirth, 2001; Gould, 2009). Natural populations of *S. aureus* isolates have shown considerable genetic heterogeneity, associated with variable strains in certain domains that are responsible for the emergence of different epidemiologic profiles (Abdulghany and Khairy, 2014). Therefore, in epidemiological studies, several molecular methods have been used for the identification and comparison of *S. aureus* isolates, including phage typing, multilocus enzyme electrophoresis, plasmid profiling, pulsed field gel electrophoresis, random amplified polymorphic DNA, ribotyping, polymerase chain reaction (PCR) of coagulase (*coa*) and protein A (*spa*) genes, and PCR-restriction fragment length polymorphism (RFLP)-based typing of *coa* and *spa* genes (Zadoks et al., 2002; Rabello et al., 2007; Mehdiratta et al., 2009; Okolie et al., 2015). Among the mentioned techniques, *coa* gene typing is a simple
and efficient method, which shows high specificity in the identification and discrimination of coa-positive S. aureus isolates (Aarestrup et al., 1995; Rodrigues da Silva and da Silva, 2005; Momtaz et al., 2011; Dallal et al., 2016; Shin et al., 2016). In the analysis of DNA sequence in the 3′-end region of coa gene, heterogeneity was detected in the 81-bp tandem SSRs region that could differ both in the number of tandem repeats and the location of AluI restriction sites among different isolates encoding 27 amino acid sequences in the C-terminus. After the amplification of the coa gene that encompasses this region, fragments of different lengths could be obtained depending on the number of repeats, which could be differentiated with AluI restriction enzyme (Goh et al., 1992). With this background in mind, the current study presents a precise and simple S. aureus genotyping method, which can be applied in epidemiological studies and infection control programs (Himabindu et al., 2009; Saei and Ahmadi, 2012). The purpose of this study was to determine S. aureus subtypes, isolated from bovine mastitis and dairy product samples, using coa gene polymorphism profile in Ahvaz, Iran.

MATERIAL AND METHODS

**Bacterial isolates.** In total, 91 isolates of S. aureus were collected from different regions of Ahvaz, Khuzestan province, Iran. Among these, 58, 9, and 24, isolates were obtained from raw cow milk, traditional food products, such as Sambooseh (n=7), and Falafel (n=2), and subclinical bovine mastitic milk samples, in different regions of Ahvaz, respectively.

**Biochemical profile.** All the isolates were subcultured three times at 37°C in a Brain Heart Infusion Broth (Merck, Germany) for 24 h. They were examined twice and reevaluated if there were any discrepancies.

**DNA extraction and coa PCR.** To prepare DNAs, a genomic DNA purification kit (Fermentas, Germany) was used, as recommended by the manufacturer. The coa gene was amplified using primers previously described by Hookey et al. (1998). The primer sequences are presented in Table 1. The PCR reaction was carried out in a total volume of 25 µl, consisting of 1.50 µl of each primer (10 pmol), five µl of DNA, 0.50 µl of dNTP mixture (10 mM each), 1.50 µl of MgCl2 (25 mM), 2.50 µl of PCR buffer (10X), and 0.50 µl of Taq DNA polymerase (1.25 units) (SinaClon Bioscience, Iran). For amplification, the following cycling parameters were applied: initial denaturation at 95°C for five min, followed by denaturation for 45 sec at 95°C, annealing for 25 sec at 57°C, extension for 25 sec at 70°C in 30 cycles, and a final five-min extension at 72°C in a mastercycler gradient thermocycler (Eppendorf, Germany).

**RFLP-PCR.** Restriction analysis of PCR products was performed with AluI enzyme (Fermentas, USA), as described by the manufacturer: 10 µl of PCR products, one µl of rapid digest enzyme, three µl of rapid digest buffer (10X), and 16 µl of nuclease-free water at 37°C for 20 min.

**Agarose gel electrophoresis.** For separating the PCR products and digested fragments, 1% and 2% agarose gel electrophoresis (Fermentas, Germany) were performed, respectively at 90 V for 30 min with 0.50 µg ml⁻¹ of safe stain (SinaGen, Iran). DNA fragments were visualized under ultraviolet light (UVitec, UK), and a 100-bp marker (Fermentas, Germany) was used as the standard for determining the size of coa and AluI-generated coa fragments.

**Specificity test.** For determining the specificity of primer pair test, DNA of S. aureus COL strain and S. epidermidis ATCC 12228 were used.

**Reproducibility test.** Five random isolates were examined over five consecutive days to evaluate PCR reproducibility. The reproducibility of RFLP-PCR was evaluated twice by AluI digestion of four products.

**Data analysis.** For determining the size of RFLP and PCR fragments, a standard curve was plotted using the distance passed through the gel and by the fragments of defined length (molecular-weight size marker). PCR genotypes and RFLP patterns were assigned numerical
codes (Table 3). For statistical analysis, Chi square test was performed in SPSS software (version 19.0) (SPSS Inc., Chicago, USA).

### RESULTS AND DISCUSSION

PCR amplification of coa gene with primers COAG2 and COAG3, showed that out of 91 S. aureus, 80 (87.91%) isolates were coa-positive (Table 2). Among them, 55 (94.82%), 21 (87.50%), 4 (57.14%), and 0 (0.00%) samples of raw cow milk, subclinical bovine mastitic milk, Sambooseh, and Falafel contained coa-positive S. aureus isolates, respectively. For most isolates (n=77, 96.25%), a single band was yielded in coa PCR with an approximate molecular size ranging from 370 to 830 bp. On the other hand, three isolates (3.75%) gave two amplification products (Figure 1). A PCR product of an estimated size of 590 bp was the most frequently detected for 48 (60.00%) isolates. Whereas, 370 and 830 bp products were the least frequent presented in two (2.50%) and one (1.25%) isolate, respectively (Table 2). The AluI RFLP patterns relative to the frequency of PCR products and genotypes are presented in Tables 2 and 3, respectively. Some PCR products generated a single pattern; however, four products (750, 670, 590, and 510 bp) generated three or more patterns. The agarose gel analysis of AluI RFLP patterns showed 23 different genotypes. Types 13, 14, 15, and 19 were the most frequent, accounting for 61.25% of the isolates. The 830, 750, 670, 670-750, 590, 590-510, 510, 510-670, and 370 bp amplicons generated different numbers of fragments, ranging from one to three (estimated size from 90 to 450 bp) (Figure 2). Moreover, 19 (90.47%) isolates from subclinical bovine mastitic milk samples contained one fragment (300 bp) and were classified as type 15; 2 (9.52%) other isolates after AluI digestion gave two fragments (450 and 220 bp) and were classified as type 12. In addition, two (50.00%) isolates from Sambooseh samples gave three fragments (220, 150, and 100 bp) and were classified as type nine; furthermore, two other isolates were classified as type 16 (Table 3). No amplification product was obtained from S. epidermidis DNA using the primers for coa gene. The PCR products were completely reproducible despite some variations in band intensity. The PCR fragments of two isolates were not digestible by AluI; therefore, this method showed a confidence of 97.50%.

The product coa gene is a main virulence factor for S. aureus strains (Goh et al., 1992). It consists of three different regions, such as N-terminus including the prothrombin-binding site, a highly conserved central region, and C-terminus, encoding 27 amino acid residues repeats (Janwithayanuchit et al., 2006). The 3′ end region of coa gene consists of tandem SSRs (81 bp). The variability of this region is the basis for the development of an easy and precise typing method of isolates from various sources (Goh et al., 1992; Aarestrup et al., 1995). Many different genotypes were detected among the studied isolates using this molecular method, which suggest that S. aureus has considerable heterogeneity in the sampled region. In the present investigation, S. aureus subtypes isolated from different sources in Ahvaz, Iran, were identified via PCR-based typing techniques (coa gene typing and PCR-RFLP). According to the current study, coa gene was detected in 87.91% of S. aureus isolates. Nine coa PCR types, ranging from 370 to 830 bp, were also detected, with one consisting of more than 59% of isolates (Table 2). Therefore, in the studied region, most cases of bovine mastitis and food samples may be caused by S. aureus strains with the same coa genotype. These findings are consistent with the observations of previous studies (Coelho et al., 2009; Saei et al., 2009; Khichar et al., 2014). Nevertheless, the other researchers have reported wider ranges of coa PCR products, such as 484 to 1080 bp (Da Silva et al., 2006), 500 to 1400 bp (Karahan and Cetinkaya, 2007), and 579 to 1442 bp (Rodrigues da Silva and da Silva, 2005). Overall, in 96.25% of the isolates, single-band

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COA</td>
<td>COAG2: 5′- ATAGAGATGCTGGTACAGG-3′</td>
<td>370-830</td>
</tr>
<tr>
<td></td>
<td>COAG3: 5′- GCTTCCGATTGTTCGATGC-3′</td>
<td>370-830</td>
</tr>
</tbody>
</table>
for coa PCR was observed, whereas in 3.75% of coa-positive isolates, double-band products were identified. In previous studies, *S. aureus* strains, which were isolated from bovine milk samples, gave single-band
amplification products (Raimundo et al., 1999). However, *S. aureus* strains from bovine mastitis have also given two PCR fragments for *coa* gene in several countries (Rodrigues da Silva and da Silva, 2005; Karahan and Cetinkaya, 2007). In this regard, (Goh et al. 1992) examined double-band products with various allelic *coa* gene forms (Goh et al., 1992). After the enzymatic digestion with AluI on *coa*-positive PCR products, 23 different genotypes were observed in the RFLP analysis. In other studies performed outside Iran, six to 49 AluI restriction patterns have been detected in bovine *S. aureus* isolates (Aarestrup et al., 1995; Raimundo et al., 1999; Rodrigues da Silva and da Silva, 2005; Karahan and Cetinkaya, 2007). Our findings showed that two types (13 and 15) are the predominant profiles among *S. aureus* strains in the study area. Overall, 19 strains (23.75%) related to genotype 15 contained one band of 300 bp, and 17 strains (21.25%) of genotype 13 consisted of three separate bands of 300, 200, and 90 bp (Table 3). These profiles seem to predominantly contribute to *S. aureus* pathogenicity in mastitis and food poisoning. In this

### Table 3. Frequency of coagulase (*coa*) genotypes in the isolated *S. aureus*

<table>
<thead>
<tr>
<th>Type code</th>
<th>Number of isolates</th>
<th>Genotype</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>830</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>750*</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>750</td>
<td>3.75</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>750</td>
<td>2.50</td>
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<tr>
<td>5</td>
<td>1</td>
<td>670</td>
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<td>7</td>
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</tr>
<tr>
<td>10</td>
<td>1</td>
<td>670-750</td>
<td>1.25</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>590*</td>
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</tr>
<tr>
<td>12</td>
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</tr>
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<td>590</td>
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<td>15</td>
<td>19</td>
<td>590</td>
<td>23.75</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
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</tr>
<tr>
<td>17</td>
<td>1</td>
<td>590</td>
<td>1.25</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>590-510</td>
<td>1.25</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>510</td>
<td>8.75</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>510</td>
<td>3.75</td>
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<tr>
<td>21</td>
<td>1</td>
<td>510</td>
<td>1.25</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
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<td>1.25</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>370</td>
<td>2.50</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td></td>
<td>100.00</td>
</tr>
</tbody>
</table>

*Not digested by AluI
*PCR= polymerase chain reaction
*RFLP= restriction fragment length polymorphism
study, a remarkable heterogeneity was observed for coa
gene of strains isolated in Ahvaz, Iran. Our results are
in line with those obtained by the other authors. Saei et
al. (2009) observed that isolates with similar and
different coa products generated different and similar
RFLP profiles, respectively. Nine PCR types and 23
AluI RFLP models showed that the isolated S. aureus
strains harbored more than one variant of coa gene.
Based on the findings, despite the presence of different
genotypes, only one or two were predominant. These
results are in line with the findings of previous research
on bovine mastitis in different countries (Güler et al.,
2005; Moon et al., 2007; Momtaz et al., 2011) and
showed that a limited number of coa genotypes were
predominant in each region and/or country. Güler et al.
(2005) typed 125 S. aureus isolates from subclinical
mastitis cow milk samples in Turkey and observed four
PCR products of 1000, 900, 800, and 700 bp, with the
1000-bp product as the predominant (Güler et al.,
2005). Moreover, Moon et al. (2007) investigated S.
aureus isolates from different sources in Korea and
identified 12 types, varying with the source of the
organisms. Nevertheless, only a few genotypes were
predominant in each source (Moon et al., 2007). Based
on the findings reported by Hennekinne et al. (2012),
SFP is one of the most common food-borne diseases
caused by the ingestion of staphylococcal heat-stable
enterotoxins produced in food by enterotoxigenic
microorganisms (Hennekinne et al., 2012). In addition,
Momtaz et al. (2011) typed 86 S. aureus isolates from
samples of bovine clinical mastitis in two provinces of
Iran and found five different genotypes. One
predominant type was found among the isolates in the
studied regions (Momtaz et al., 2011). Furthermore, in
studies conducted by Su et al. (1999) and Mullarky et
al. (2001), microorganisms with the dominant coa
genotypes showed greater resistance to the host
immune factors, such as neutrophil activity, compared
to those with rare genotypes. Therefore, the dominant
types might have particular characteristics, which can
help to overcome the host defense mechanisms (Su et
al., 1999; Mullarky et al., 2001). This study indicated
that coa gene analysis could be beneficial for typing S.
aureus strains isolated from different sources, such as
bovine mastitis and dairy products. Furthermore,
although the results showed that the samples in the
studied region were infected with S. aureus strains
harboring more than one coa genotype, only one or two
genotypes were predominant. Further research is
recommended to identify the features of dominant
strains using greater number of strains. Such studies
could help present control measures for staphylococcal
mastitis and dairy food contamination by S. aureus.

Ethics
We hereby declare all ethical standards have been
respected in preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

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Chamran University of Ahvaz, Ahvaz, Iran.

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